Microscale Topography Therapy for Type 2 Diabetes: Diabetic Macrophages Reprogrammed with Microtextured Topography

Thesis

Presented in Partial Fulfillment of the Requirements for Graduation with Honors Research Distinction at The Ohio State University

by

Shipeng Wang
Undergraduate Program in Biomedical Engineering
The Ohio State University
2020

Thesis Committee
Daniel Gallego-Perez, Advisor PhD
Silvia Duarte-Sanmiguel

Abstract

In 2015, over 9.5% of Americans have diabetes and 95% of them are diagnosed with type 2 diabetes (T2D). [1] While existing treatments are not effective enough, more efficient treatment is needed. Macrophage is a type of white blood cell that plays a crucial role in the immune system by digesting foreign substances. It can differentiate into two phenotype macrophages: M1 "killing" phenotype that can exacerbate T2D condition and M2 "healing" phenotype that can ease T2D symptoms.[4] Researchers have found out that under T2D condition, the number of M1 phenotype macrophages dominates over that of M2 phenotype. Thus, a possible treatment for T2D is to find ways to shift M1phenotype to M2 phenotype. While the macrophage can polarize and repolarize to different phenotypes depending on different stimulations, we found that M1 macrophage can polarize to M2 phenotype on the microtextured culture. [4] Thus, the objectives of this project are to test the efficacy of M2 polarization with microtextured culture. This project will utilize micro-patterned topography to reprogram macrophages from diabetic mice in vitro and probe the mechanism of the shift. Then we will evaluate the potency of such treatment using flow cytometry, western blot and real-time Polymerase Chain Reaction (PCR).

Acknowledgements

I would like to thank my principle investigator, Dr. Gallego-Perez, and my research mentor, Silvia Duarte-Sanmiguel, for offering me the opportunity to join the lab as a volunteer and pointing out the topic for my research distinction; for their diligent guidance that inspired my passion towards biomedical engineering and pointed out the right way for me when I was confused during the experiments; and for their high expectations that granted me confidence of myself and pushed me forward every single day. In addition to research, I have learned so much from you, and for this I cannot thank you enough. I have truly enjoyed seeing our lab grow since I joined in, I look forward to reading about all the great works you accomplish in the future.

I would also like to thank all of the people working under Dr. Gallego-Perez's and Dr. Higuita-Castro's labs, for their enthusiasm of teaching me how to use all the equipment, providing advice when I was confused, and most importantly, making me feel at home. I sincerely wish success for all of you in the future.

To my parents, Jianwen Wang and Jiuli Gong, I am grateful for your many years of guidance and support. I am who I am because of all of you, someone who is driven to be successful and make a difference in the world.

And my grandma, thank you for your inspiration and motivation.

This work was supported through funding from the College of Engineering Honors Research Committee at The Ohio State University.

Thank you all, it has truly been an honor.

Vita

Education

2016-2020 B.S Biomedical Engineering

The Ohio State University

Research Experience

2019 Research Assistant to Dr. Gallego-Perez

Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University

2019 Research Volunteer to Dr. Gallego-Perez

Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University

2016 Research Volunteer to Dr. Peng

Department of Life Science, Sichuan University

Honors and Awards

2019-20 Honors Research Scholarship, College of Engineering

The Ohio State University

Fields of Study

Major Field: Biomedical Engineering

Table of Contents

List of Figures

List of Tables

Chapter 1. Introduction

Type 2 Diabetes is a common chronic disease associated with insulin resistance of cells in the body. When the digestive tract absorbs glucose in food, the glucose enters the blood vessels and thus increases the blood sugar level. The rise of glucose concentration in blood is then sensed by beta cells located in the pancreas. In order to maintain the homeostasis, those beta cells secrete insulin, which can be received by insulin receptors on the surface of cells in the body. The activation of insulin receptors enables cells to absorb the glucose from the blood for activities and thus decreases the blood sugar level. In the case of T2D, the receptors on other cells of the body are malfunctioning, and therefore the glucose level in blood cannot be regulated, leading to hyperglycemia. Long term of hyperosmolar hyperglycemic state might cause obesity, cardiovascular diseases and kidney failures, which makes it the 7th leading cause of death in the U.S [1].

Over the past several decades, treatments have been invented for T2D. Some focus on behavioral treatment such as exercise and diet, others require oral or injectable medications. While a change of lifestyle is a long and complicated progress, medicine especially metformin can increase insulin sensitivity of cells, slow down the absorption of glucose from food and slow down the release of glucose in the liver [2]. However, those treatments can be either time-consuming or palliative [3]. Since there is still a huge number of T2D patients, there is an urgent need to discover new effective treatments.

Macrophages are important cells of the immune system. Associated with obesity, they can be found accumulated in adipose tissue (visceral fat) and bone marrow [11]. A research shows that macrophages can acquire different phenotypes such as M1 and M2 depending on the

microenvironment [4]. M1 (pro-inflammatory) can trigger Th1 immune response and kill microorganisms if exposed to specific signals, but it also increases insulin resistance. M2 (anti-inflammatory), on the other hand, helps tissue and vascular to repair by triggering Th2 immune response and decreases insulin resistance [4]. Interestingly, M1 and M2 phenotypes have been found closely related to T2D: M1 numbers and M1/M2 ratio are associated with insulin resistance [5]. And since insulin resistance is one of the most important factors correlated to T2D, it is possible to find a way to cure T2D by manipulating the ratio of M1/M2.

Microscale topography, a technology that modifies surfaces with microscale texture, has been used frequently in various fields of science. Contact guidance is the phenomenon where cells contact with their microenvironments and thus adjust the cytoskeleton, which changes the behavior, morphology, or even phenotype depending on the texture or structure of the microenvironment they make contact with. Besides soluble factors presented in the microenvironment, research also finds that the surface texture where macrophages attached to is also important for the polarization of macrophages [6]. More specifically, elongated shape and groove with a particular width can trigger contact guidance phenomenon that leads to the expression of M2 phenotype in healthy macrophage [6,7]. However, currently there is no research associating macrophage polarization with T2D treatment. Hence, using topography to control the M1/M2 ratio of T2D macrophage could be a potential solution to treat T2D.

ROCK-1/2 are proteins found to play roles in macrophage polarization by regulating the cell skeleton, glucose and fatty acid transport, and NFκβ phosphorylation [8,9,10]. Before jumping into in vivo study of how the reprogrammed macrophages could affect the T2D, we decided to investigate the underlining mechanism of how ROCK-1/2 stimulated by topography

could impact the glucose and fatty acid transporters by inhibiting the ROCK-1/2 proteins in macrophages and evaluating the M1/M2 markers after treatments [12].

Micro-topography is relatively new microtechnology using neither biological nor chemical approaches to treat diseases. Compared to medical treatment for T2D, micro-topography might have less to no side effects on the body, and it might be able to treat T2D once and for all. It is also a more practicable method compared to changing of lifestyles. The ability to manipulate the M1/M2 ratio could be utilized to treat other immune system problems as well since M1 and M2 macrophages have lots of other functions too. Since this therapy will use PBMCs from the patient for M2 polarization, there will be no immune rejection after injecting M2 cells back to the patient. The therapy could also be designed for different patients under different conditions for different outcomes too. We hypothesize that the micro-patterned topography will stimulate ROCK-2 production, which polarizes the macrophage into M2 phenotype.

Chapter 2. Materials and Methods

2.1 Patterned PDMS preparation

Silicon wafers with micro-patterned (2μm wide, 1.5μm tall, spaced by 2μm) were made according to standard photolithography technique using S1813 photoresist. Curing base and curing agent (Sylgard 184) were mixed with a ratio of 10:1 with a total mass of 16g per Polydimethylsiloxane (PDMS) plate to be made. The mixture was put into a vacuum for 10 minutes to remove the air bubbles appeared during mixing. Silicon wafer with micro-pattern was placed in the center of a spinner and fixed by vacuum. 6mL of curing mixture were added on the surface of the wafer. The wafer was spun at 300 RPM for 1 minute. The wafer with PDMS was then placed in a 15mL petri dish. The dish was then sent to incubate at 65°C for 2 hours or incubate at room temperature for 48 hours. The solidified PDMS plate was then carefully removed from the silicon wafer. This process is shown in Figure 1.

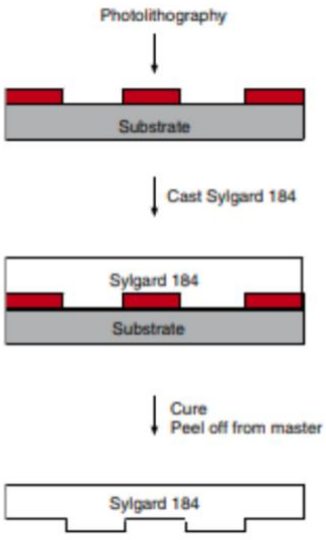

Figure 1: Soft lithography of PDMS [13]

Six circles with the size smaller than a well in 6-wells plate were cut and placed into 6-wells plate with the textured side facing up. The wells were washed with 2mL of phosphate-buffered saline (PBS), 2mL of 70% Ethanol, 2mL of PBS again. The liquids were aspirated. The 6-wells plate was sterilized under UV light for 2 minutes.

2.2 Animal use

The care and use of animals observed NIH guidelines and were approved by the Ohio State University Institutional Animal Care and Use Committee. Obese male mice with db/db mutation that induces type 2 diabetes via hyperphagia were used as treatment groups for this study.

2.3 Bone marrow isolation & cell culture

Mice were sacrificed with Carbon dioxide. Femurs and tibias were isolated from both hind limbs washed with PBS and placed into plain Dulbecco's Modified Eagle Medium (DMEM) on ice. Femurs and tibias were washed by PBS, 75% Ethanol and PBS. Both ends of the femurs and tibias were cut. A syringe was filled with plain DMEM. The bone marrow was removed by pressing the plain DMEM through the femurs and tibias with the syringe. The mixture of DMEM and bone marrow was resuspended until no clots were visible, centrifuged at 4°C with 1000 RPM for 10 minutes, and aspirated. 10mL of red blood cell lysis (distilled & deionized water to RBC lysis solution name ratio: 10:1) was added and incubated for 10 minutes to break the red blood cells. The solution was then centrifuged and aspirated. BSA and CD11b Microbeads were added at a ratio of 9:1 to label the targeting monocytes, the progenitor of bone marrow-derived macrophages (BMDMs) with magnetic antibodies. The solution was passed through a MS column in QuadriMACS™ separator to filter out unwanted cells. The column was then removed

from the magnetic field and 9mL of BSA was used to wash the monocytes off the column. Monocytes labeled with antibodies were collected in a separate Eppendorf tube, counted, centrifuged and aspirated. 2 million of cells were cultured with mix macrophage media (IMDM+Gluta-max, 10% inactivated FBS, 1% ANTI/ANTI, 0.1% Granulocyte-macrophage colony-stimulating factor (GM-CSF), which stimulates stem cells to produce monocytes) in each well of the 6-wells plate (with micro-patterned PMDS). Plain-patterned PDMS served as another control in comparison to micro-patterned PDMS to demonstrate the effects that micro patterns have on cells. Plate was incubated in incubator at 37 °C.

At day 3 of incubation, the old media was aspirated and fresh media was added. Cells were treated with either ROCK1 (Y-27632) at concentrations of 0μM, 50μM and 100μM or ROCK2 (KD025) at concentrations of 0μM and 25μM. Non-treated cells cultured in micro-patterned PDMS served as positive controls while cells cultured in plain PDMS served as negative controls.

2.4 Cell collection

Cells were collected at day 7 for qRT PCR, western blot. For PCR, wells were aspirated and washed with 1mL PBS. After incubation at room temperature for 5 minutes, PBS was removed and 1mL of TRizol reagent (ThermoFisher) was added. Following another 5 minutes of incubation, cells were scratched, collected in 2mL Eppendorf tube and stored at -80°C. For western blot, wells were washed with PBS, aspirated and added with 1mL of cold PBS with ethylenediaminetetraacetic acid (EDTA) After incubation at 37°C for 10 minutes, the PBS was aspirated. The cells were collected after centrifuge and aspiration.

2.5 Quantitative gene expression analysis

Total RNA was extracted from cells collected at day 7 using TRizol reagent according to manufacturer's instructions. RNA samples were used for the synthesis of cDNA using the SuperScript® VILO cDNA Synthesis Kit (ThermoFisher). Quantitative real-time PCR was performed by the QuantStudio 3 Real-Time PCR System with TaqMan fast advance chemistry (Thermo Scientific) using mouse gene-specific primers for M1and M2 macrophage markers to determine mRNA levels per cell. Primer names shown below in Table 1.

M1 markers	TNFα	IL6	CCR2	NOS2	IL1b
M2 markers	IL10	ARG1	VEGFA		
Internal controls	18S	GAPDH			

Table 1: Mouse gene-specific primers used in this study.

Tumor necrosis factor alpha (TNFα) is a cytokine mainly produced by macrophages in respond of systemic inflammation [14]. Interleukin 6 (IL6) can be found both being pro-inflammation cytokines and anti-inflammation cytokines. When IL6 is signaling in macrophage, it creates pro-inflammatory response [15]. Chemokine receptor type 2 (CCR2) encodes a receptor that involves in monocyte infiltration and inflammatory response [16]. Nitric oxide synthase (NOS2 or iNOS) can be found in monocytes or macrophages from patients with inflammatory diseases [17]. Interleukin 1 beta (IL1b) functions as a mediator of inflammatory response

For M2 markers, those markers can be found in anti-inflammatory macrophages. Interleukin 10 (IL10) is capable of inhibiting the synthesis of pro-inflammatory cytokines such as TNFα. Arginase 1 (ARG1) encodes the arginine production, which relates to the production of polyamines and collagen and favors tissue remodeling and wound healing [18]. And finally, vascular endothelial growth factor A (VEGFA) is responsible for vascularization of endothelial cells and macrophage migration.

18S is important for ribosomal function and thus frequently used for PCR. Glyceraldehyde 3-phospate dehydrogenase (GAPDH) plays a crucial role in glycolysis, and thus is considered as a housekeeping gene. Both 18S and GAPDH will serve as internal control in this study.

2.6 Statistical analysis

Data were analyzed using Excel and SPSS. We used n = 3–5 replicates per experiment. Specific information on the number replicates, and statistical tests can be found in the figure legends. The difference of cytokine expression between treatment group and control group will be analyzed using paired samples T-test. A p value less than 0.05 will be considered as significant.

Chapter 3. Results and Discussion

Before the concentrations of ROCK1/2 inhibitors were determined, the original concentrations were set as 20μM for ROCK1 and 20μM for ROCK2. However, those concentrations seemed not to have significant impacts on the fold changes and expression levels of M1 and M2 markers. Thus, instead of moving to mice injection of reprogrammed macrophages, we decided to find the most suitable concentrations for ROCK1/2 inhibitor to probe the role of ROCK1/2 in topographical reprogramming.

At day 1, the bone marrow was collected from mice femurs and tibias. By treating the bone marrow with red blood cell lysis and magnetic separator, most of the red blood cells and platelets were filtered, leaving only white blood cells. The white blood cells were stimulated by GM-CSF in media and differentiated into monocytes, which then differentiated into macrophages.

At day 3 before treatment, macrophages were found attaching to the micro-patterned surface of PDMS. Most of the cells changed from round shapes into linear shapes aligning with the micro-patterned on the surface. We could safely conclude that linear-shaped cells successfully attach to pattern whilst those float round-shaped cells are dead.

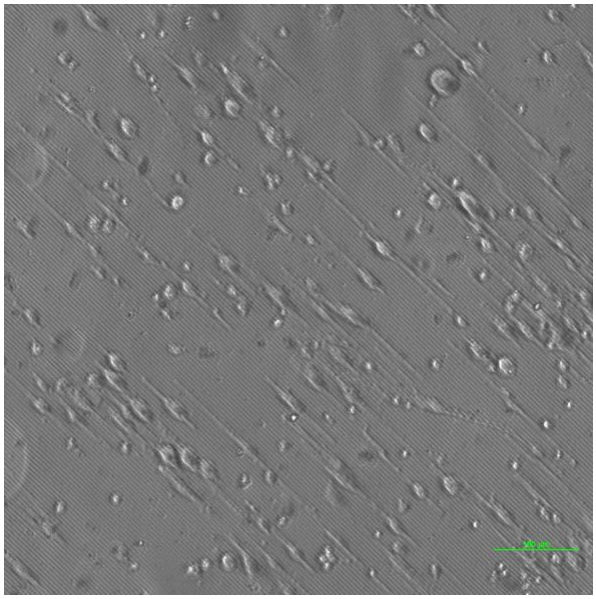

Figure 2: Image of macrophages attaching to the micro patterns.

To illustrate the fact that micro-patterned topography has changed the cell morphology, we cultured monocytes on plain-patterned PDMS as well. Macrophages grown on plain patterns had more random shapes with multiple “synapsis-liked” features while those cultured on micro-patterned topography aligned with the patterns in linear shapes via contact guidance.

By treating macrophages with ROCK-1/2 inhibitors, ROCK-1/2 proteins will be inhibited due to the inhibitors competing with ATP for binding to the catalytic sites, while the uninhibited ROCK protein will be stimulated preferentially.

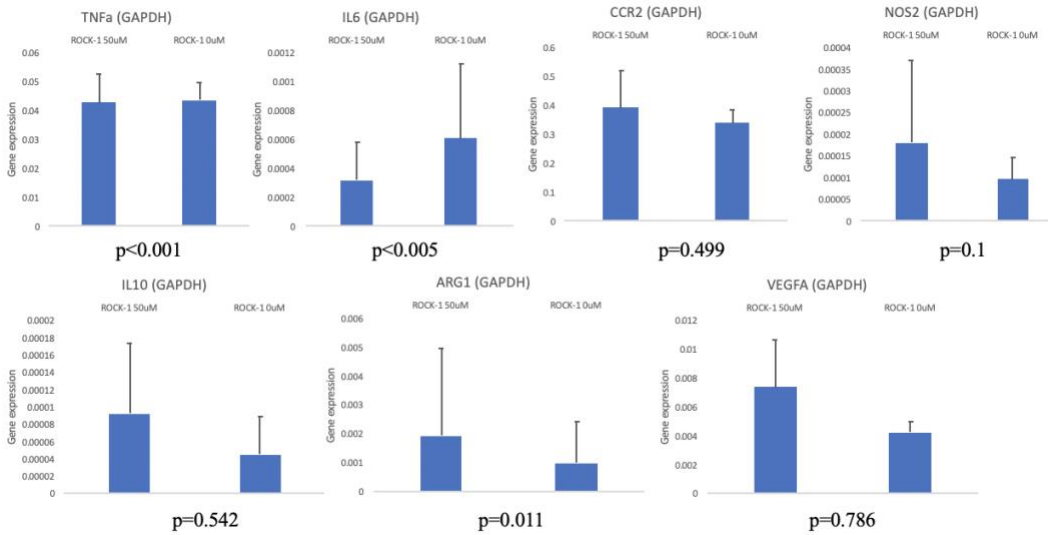

Figure 3: Quantitative PCR results in comparison to GAPDH of db/db mice macrophages treated with ROCK-1 inhibitor with a concentration of 50uM. The top four genes represented M1 markers while the lower three genes represented M2 markers. (One-tailed test, n=3 per treatment).

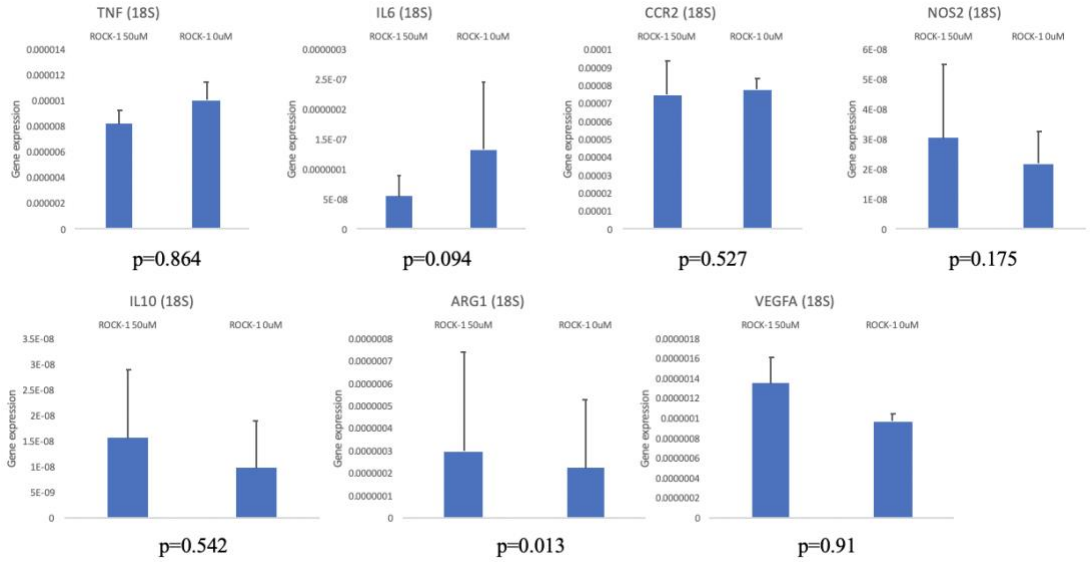

Figure 4: Quantitative PCR results in comparison to 18S of db/db mice macrophages treated with ROCK-1 inhibitor with a concentration of 50uM. The top four genes represented M1 markers while the lower three genes represented M2 markers. (One-tailed test, n=3 per treatment).

From figure 3&4, we can see an approximate trend where M2 markers expressed more in treatment groups while M1 markers expressed more in control groups. This makes sense because those macrophages were inhibited with 50uM of ROCK-1 inhibitor, which lead to ROCK-2 stimulation by the micro-topography. However, not all of the genes responded in the same manner. CCR2 and NOS2 cytokines gene expressions in macrophages treated with ROCK-1

inhibitor were higher than those in untreated control groups. The statistical analysis also revealed insignificance. One possible reason could be due to the fact that the sample size is limited at 3, which could be improved by adding more replicates. It could also because that the concentration of the ROCK-1 inhibitor was not high enough such that the differences between inhibited and uninhibited macrophage gene expressions were not significant. Thus, we decided to increase the concentration of ROCK-1 inhibitor to 100uM.

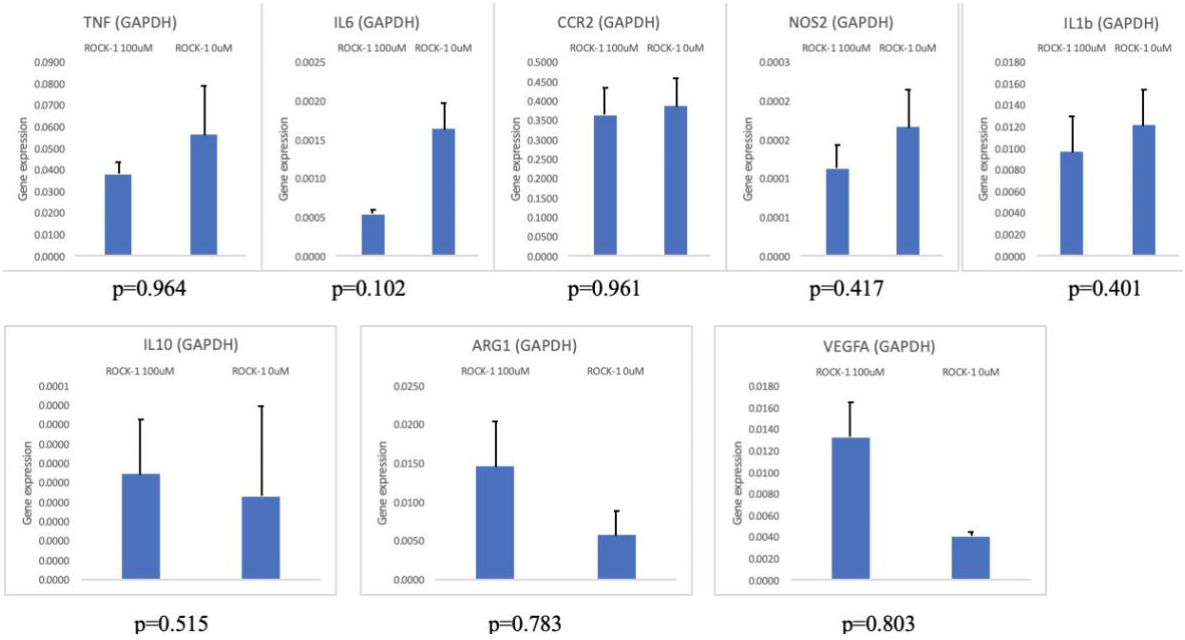

Figure 5: Quantitative PCR results in comparison to GAPDH of db/db mice macrophages treated with ROCK-1 inhibitor with a concentration of 100uM. The top four genes represented M1 markers while the lower three genes represented M2 markers. (One-tailed test, n=4 per treatment).

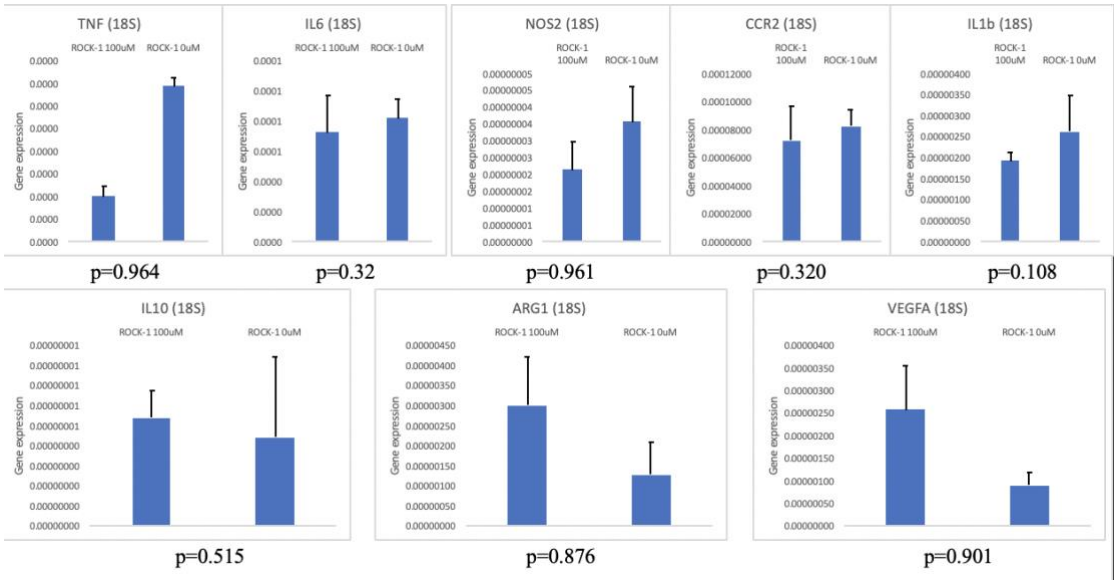

Figure 6: Quantitative PCR results in comparison to 18S of db/db mice macrophages treated with ROCK-1 inhibitor with a concentration of 100uM. The top four genes represented M1 markers while the lower three genes represented M2 markers. (One-tailed test, n=4 per treatment).

At a concentration of 100uM, the impacts that ROCK-1 inhibitor has on db/db mice macrophages became more obvious. The M1 markers expression in control group were consistently higher than those in treatment group judging from the mean gene expression levels. M2 markers also followed the same trend as we saw in 50uM ROCK-1 inhibitor treatment. As a result, 100uM of ROCK-1 inhibitor seemed to work better at investigating the role of ROCK-1/2 in topography reprogramming since all M1 markers showed similar responses that would be easier to identify when probing the role of ROCK-1/2. Combined the ROCK-1 inhibitor results, we can see that there might be correlations between the ROCK-1 inhibitor and M1 marker expressions, and between the ROCK-1 inhibitor and M2 marker expressions.

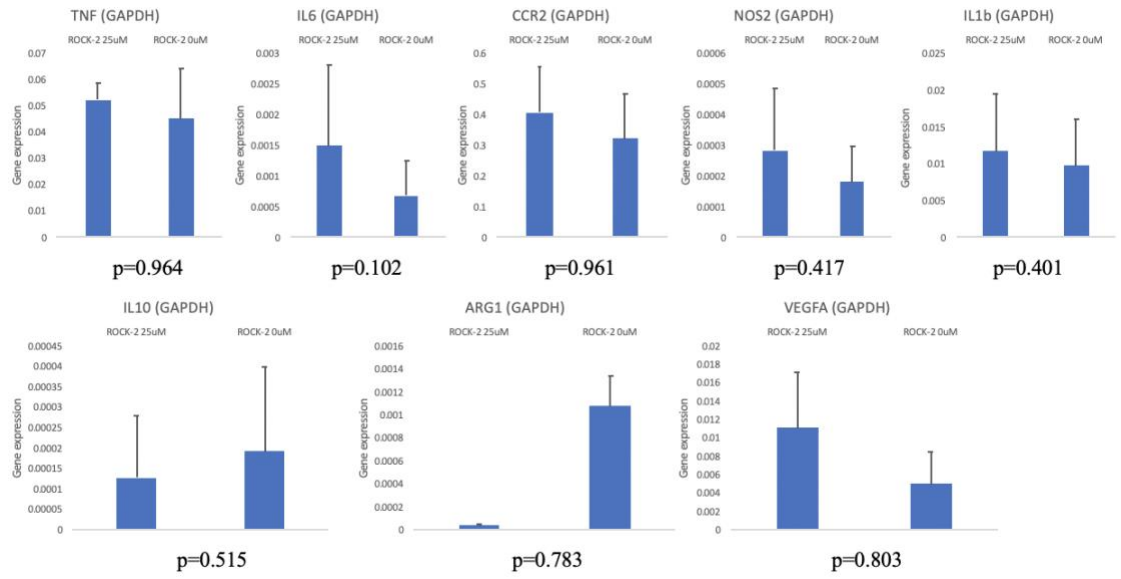

Figure 7: Quantitative PCR results in comparison to GAPDH of db/db mice macrophages treated with ROCK-2 inhibitor with a concentration of 25uM. The top four genes represented M1 markers while the lower three genes represented M2 markers. (One-tailed test, n=5 per treatment).

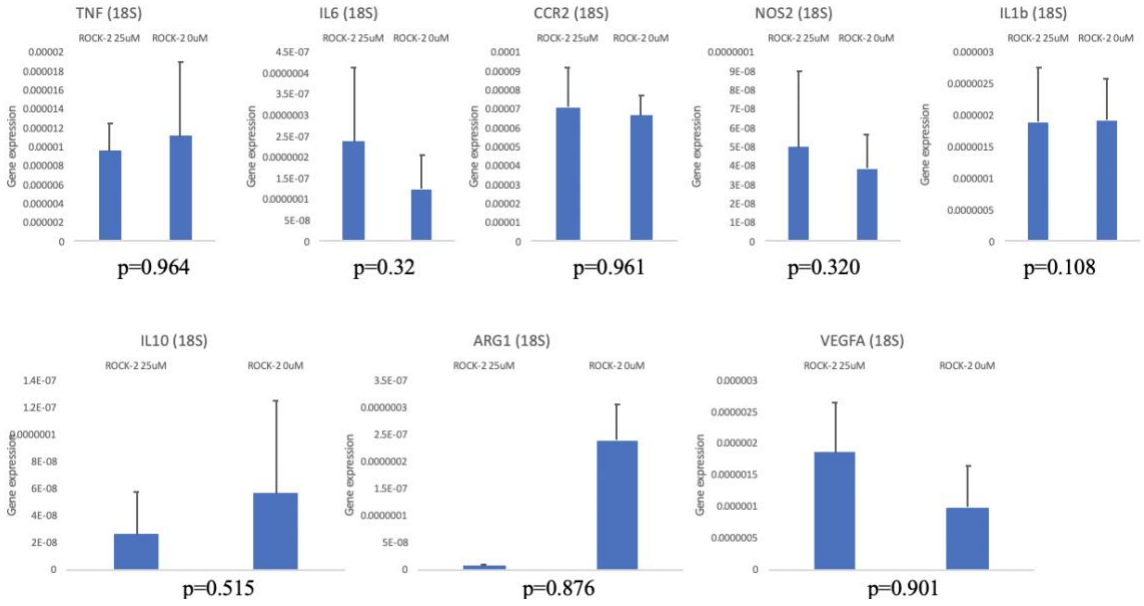

Figure 8: Quantitative PCR results in comparison to 18S of db/db mice macrophages treated with ROCK-2 inhibitor with a concentration of 25uM. The top four genes represented M1 markers while the lower three genes represented M2 markers. (One-tailed test, n=5 per treatment).

After treated with 25uM of ROCK-2 inhibitor, db/db mice macrophages showed generally higher expressions of M1 markers and lower expressions of M2 markers compared to untreated control groups with the exceptions of TNFa, and VEGFA expressions. This result demonstrated that the inhibition of ROCK-2 proteins in diabetic mice macrophages may lead to

ROCK-1 stimulation that favors glucose uptake/metabolism (M1 phenotype). However, the existence of outliers such as TNFa, and VEGFA should not be neglected. Future work should be set to investigate the relationships between those cytokines and diabetic mice macrophage phenotype.

Another thing to notice about the ROCK-2 inhibitor treatment is that the 25uM concentration seemed to be harmful for the macrophages. In figure 9, the groups treated with 25uM of ROCK-2 inhibitor had much more floating dead cells compared to the untreated control groups, which indicated that such concentration might kill macrophages. Study has found that ROCK-1 is involved in destabilizing actin cytoskeleton while ROCK-2 is required for stabilizing actin cytoskeleton, which further explains the reason why ROCK-2 inhibitor at much lower concentration could kill cells: the inhibition of ROCK-2 will destabilize actin cytoskeleton, which is crucial for macrophages to attach to the micro-patterned PDMS [19]. Thus, it's necessary to decrease the concentration of ROCK-2 inhibitor in the future study.

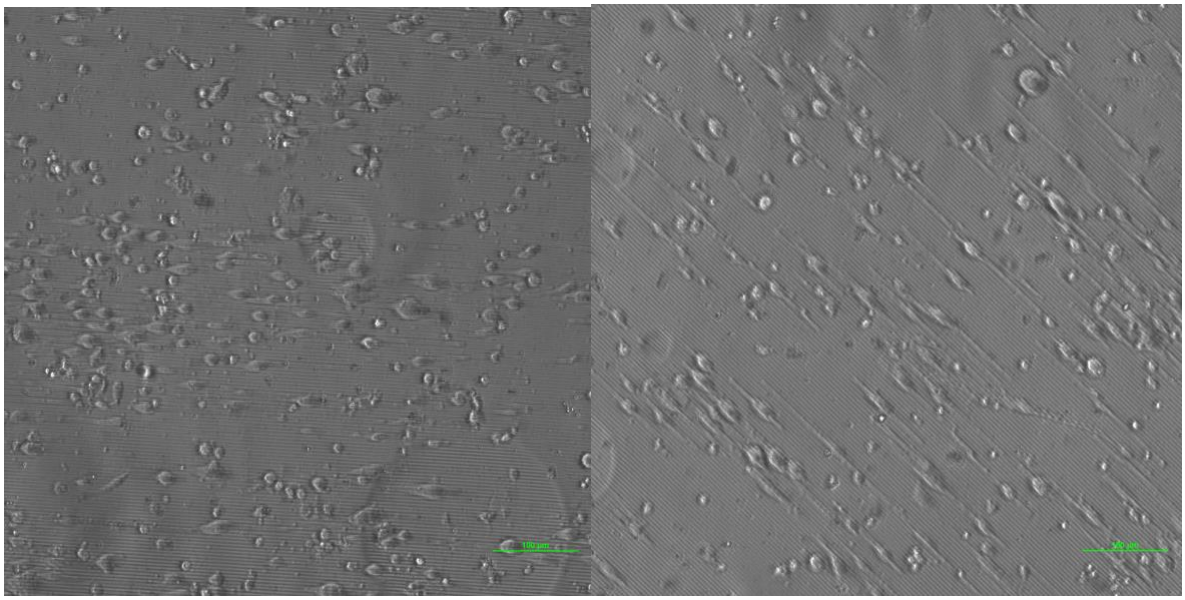

Figure 9: Microscopic images of diabetic mice macrophages treated with ROCK-2 25uM inhibitor (left) and control group (right).

The image on the left has more round cells.

As the statistics indicated for each cytokine expression level, most of the p values are not significant. The major reason I could think of is the fact that the sample size is really small, with macrophages collected from 1 to 2 mice per treatment. Thus, the results might remain inconclusive until further work is done.

Chapter 4. Conclusion

4.1 Summary

The purpose of this study is to probe the role of ROCK-1/2 in diabetic mice macrophages reprogrammed by micro-patterned topography. Our hypothesis is that at low level of ROCK-1 proteins, the micro-patterned topography could stimulate the ROCK-2 proteins and thus leading to a M2 phenotype. To investigate the role, we treated the macrophages with ROCK-1/2 inhibitor at different concentration separately to see how the micro-patterned topography could help stimulate the uninhibited ROCK protein and evaluated the M1 & M2 markers expressions of the cells to speculate the outcomes.

As a result, while ROCK-1 50uM inhibitor might not generate a good outcome for the investigation of the role of ROCK-1/2, ROCK-1 100uM inhibitor and ROCK-2 25uM inhibitor appeared to be effective for us to probe the role of ROCK-1/2 in topographically stimulated macrophages as the M1 and M2 markers tent to have more consistent results. We could see that compared to the untreated control group, the macrophages treated with ROCK-1 100uM inhibitor had higher expression of M2 markers. This may demonstrate the fact that at low ROCK-1 levels, micro-patterned topography could stimulate ROCK-2 production, leading to a M2 phenotype. However, we still need to test the levels of ROCK-1/2, GLUT-1 and CD36 to make a conclusion.

Diabetic mice macrophages treated with ROCK-2 25uM inhibitor yielded the opposite outcome, where treatment group had higher M1 markers expressions than control group. This might illustrate that the inhibition of ROCK-2 proteins in diabetic mice macrophages may lead to

ROCK-1 stimulation. However, we can't make any conclusions about the ROCK-2 stimulation by micro-patterned topography in this case. The concentration of ROCK-2 inhibitor was so high that it may have damaged the actin cytoskeleton and thus killed the macrophages according to the result.

In conclusion, ROCK-1 inhibitor at a concentration of 100uM could be useful for the stimulation ROCK-2 production via micro-patterned topography. This may indicate that ROCK-1 proteins at lower concentration could help to increase the stimulation of ROCK-2 by micro-patterned topography, and thus leading to a M2 macrophage phenotype.

4.2 Limitations

Due to time limitation, this study has only finished part of the original experiment. Sample size, for example, was three to five, which is considered small to generate statistically significant results. We will need to run western blot to detect the protein levels of ROCK-1/2, GLUT-1 and CD36 and flow cytometry to evaluate the phenotype of macrophages after inhibition. Another limitation is the lack of samples cultured on plain patterns, which could be used to probe the effects inhibitors have on cells. In other words, we need to demonstrate that it was the micro-patterned topography that stimulated the ROCK-2 proteins when macrophages were treated with inhibitors. In addition, the throughput of the bone marrow isolation process is low. Each mouse could only provide 4 to 6 wells with each well having around 2 million cells, which is required for macrophages to grow since they live in high confluency environment naturally.

4.3 Future work

To better test the hypothesis that the micro-patterned topography could stimulate the ROCK-2 proteins and thus leading to a M2 phenotype, further tests to identify the protein levels as well as macrophage phenotype are needed. In addition, it's important to include another control group where having plain-patterned PDMS will serve as the negative control, while having inhibitors as positive control to ensure that it is the micro-patterned topography that is stimulating the macrophages to express more ROCK-2 genes. Future work is also needed to improve the protocol of bone marrow isolation so that the throughput would be higher.

As the hypothesis of this study is tested, our next step would be to test the M2 macrophages reprogrammed by micro-patterned topography based on the findings of this study by injecting those cells back to the visceral fat compartment of db/db mice to evaluate how reprogrammed cells will shift the M1/M2 ratio in vivo and thus affect the outcome.

4.4 Contribution

The silicon wafer with micro-patterned topography was manufactured by NanoTech West. The bone marrow isolation and cell culture protocols were developed by Silvia Duarte-Sanmiguel and Daniel Gallego-Perez, PhD. I designed the experiment, made the PDMS, isolated the bone marrow, collected the cells, ran the qPCR and analyzed the data. Guidance for this project was provided by Silvia Duarte-Sanmiguel, Daniel Gallego-Perez, PhD.

Bibliography

1. Berry, Jennifer. (2019). Statistics and facts about type 2 diabetes. *Medical News Today.* https://www.medicalnewstoday.com/articles/318472.php

2. Burgess, Lana. (2019). Can people with type 2 diabetes stop taking metformin? *Medical News Today.* https://www.medicalnewstoday.com/articles/323128.php

3. Marín-Peñalver, Juan José, et al. "Update on the Treatment of Type 2 Diabetes Mellitus." *World Journal of Diabetes*, Baishideng Publishing Group Inc, 15 Sept. 2016, www.ncbi.nlm.nih.gov/pmc/articles/PMC5027002/.

4. Fraternale A, Brundu S & Magnani M. (2015). Polarization and Repolarization of Macrophages. *Clinical & Cellular Immunology.*

5. Fujisaka, Shiho, et al. "Regulatory Mechanisms for Adipose Tissue M1 and M2 Macrophages in Diet-Induced Obese Mice." *Diabetes*, American Diabetes Association, Nov. 2009, www.ncbi.nlm.nih.gov/pmc/articles/PMC2768159/.

6. McWhorter, Frances Y., et al. "Modulation of Macrophage Phenotype by Cell Shape." *PNAS*, National Academy of Sciences, 22 Oct. 2013, www.pnas.org/content/110/43/17253.

7. Luu, Thuy U., et al. "Micro- and Nanopatterned Topographical Cues for Regulating Macrophage Cell Shape and Phenotype." *ACS Publications*, 2015, pubs.acs.org/doi/abs/10.1021/acsami.5b10589.

8. Zandi, S. et al. ROCK-isoform-specific polarization of macrophages associated with age-related macular degeneration. *Cell reports* **10**, 1173-1186 (2015).

9. Chun, K.-H. et al. Regulation of glucose transport by ROCK1 differs from that of ROCK2 and is controlled by actin polymerization. *Endocrinology* **153**, 1649-1662 (2012).

10. Zhou, Q. et al. ROCK2 Deficiency in Bone Marrow-Derived Cells Leads to Increased Cholesterol Efflux and Decreased Atherosclerosis. *Circulation* **126**, 2236 (2012).

11. Marim, F.M., Silveira, T.N., Lima Jr, D.S. & Zamboni, D.S. A method for generation of bone marrow-derived macrophages from cryopreserved mouse bone marrow cells. *PloS one* 5, e15263 (2010).

12. Nishimaru, Kazuhide, et al. "Inhibition of Agonist-Induced Positive Inotropy by a Selective Rho-Associated Kinase Inhibitor, Y-27632." *Journal of Pharmacological Sciences*, vol. 92, no. 4, 2003, pp. 424–427., doi:10.1254/jphs.92.424.

13. Qin, D., Xia, Y. & Whitesides, G. M. Soft lithography for micro- and nanoscale patterning. *Nat. Protoc.* **5,** 491–502 (2010).

14. Parameswaran, Narayanan, and Sonika Patial. "Tumor Necrosis Factor-α Signaling in Macrophages." *Begell House*, vol. 20, no. 2, 2010, doi:10.1615/CritRevEukarGeneExpr.v20.i2.10.

15. Brandt, Claus, and Bente K. Pedersen. "The Role of Exercise-Induced Myokines in Muscle Homeostasis and the Defense against Chronic Diseases." *Journal of Biomedicine and Biotechnology*, vol. 2010, 2010, pp. 1–6., doi:10.1155/2010/520258.

16. Willenborg, Sebastian, et al. "CCR2 Recruits an Inflammatory Macrophage Subpopulation Critical for Angiogenesis in Tissue Repair." *Blood*, vol. 120, no. 3, 2012, pp. 613–625., doi:10.1182/blood-2012-01-403386.

17. Weigert, Andreas, and Bernhard Brüne. “Nitric Oxide, Apoptosis and Macrophage Polarization during Tumor Progression.” *Nitric Oxide*, vol. 19, no. 2, 2008, pp. 95–102., doi:10.1016/j.niox.2008.04.021.

18. Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol*. (2013) 229:176–85. doi: 10.1002/path.4133

19. Shi, Jianjian, et al. “Distinct Roles for ROCK1 and ROCK2 in the Regulation of Cell Detachment.” *Cell Death & Disease*, vol. 4, no. 2, 2013, doi:10.1038/cddis.2013.10.